Rhizobium meliloti Genes Involved in Sulfate Activation: The Two Copies of *nodPQ* and a New Locus, *saa*

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ABSTRACT

The nitrogen-fixing symbiont *Rhizobium meliloti* establishes nodules on leguminous host plants. Nodulation (*nod*) genes used for this process are located in a cluster on the pSym-a megaplasmid of *R. meliloti*. These genes include *nodP* and *nodQ* (here termed *nodPQ*), which encode ATP sulfurylase and APS kinase, enzymes that catalyze the conversion of ATP and SO_4^{2-} into the activated sulfate form 3′-phosphoadenosine 5′-phosphosulfate (PAPS), an intermediate in cysteine synthesis. In Rhizobium, PAPS is also a precursor for sulfated and *N*-acylated oligosaccharide Nod-factor signals that cause symbiotic responses on specific host plants such as alfalfa. We previously found a highly conserved second copy of *nodPQ* in *R. meliloti*. We report here the mapping and cloning of this second copy, and its location on the second megaplasmid, pSym-b. The function of $nodP_2Q_2$ is equivalent to that of $nodP_1Q_1$ in complementation tests of *R. meliloti* and *Escherichia coli* mutants in ATP sulfurylase and adenosine 5′-phosphosulfate (APS) kinase. Mutations in $nodP_2Q_2$ do not have as severe an effect on symbiosis or plant host range as do those in $nodP_1Q_1$, however, possibly reflecting differences in expression and/or channeling of metabolites to specific enzymes involved in sulfate transfer. Strains mutated or deleted for both copies of *nodQ* are severely defective in symbiotic phenotypes, but remain prototrophic. This suggests the existence in *R. meliloti* of a third locus for ATP sulfurylase and APS kinase activities. We have found a new locus *saa* (sulfur amino acid), which may also encode these activities.

RHIZOBIUM meliloti is a symbiotic soil bacterium that can induce nodule formation on and invade the roots of *Medicago sativa* (alfalfa) and some other legumes. The bacteria interact with and infect epidermal plant cells, stimulate nodule morphogenesis and invade these plant nodule cells through an infection thread. The bacteria are released into the nodule cells, where they differentiate into nitrogen-fixing bacteroids (LONG 1989a; DE BRUIJN and DOWNIE 1991; see also STACEY, BURRIS and EVANS 1991; VERMA 1991; BREWIN 1991).

The "symbiotic" genes in the bacterium are needed for all the stages of this process. These include *nod* genes, involved in early stages of the host's response; *exo* genes, which affect surface exopolysaccharides and nodule invasion by the bacteria; and *nif* and *fix* genes, required for nitrogen fixation by the bacteriods. Many of these genes are located on either pSyma or pSymb, the two megaplasmids of *R. meliloti* (reviewed by LONG 1989b). pSyma contains *nod*, *nif* and *fix* genes (BANFALVI, KONDOROSI and KONDOROSI 1985; BROMFIELD *et al.* 1987; BURKARDT and BURKARDT 1984; BURKARDT, SCHILLIK and PÜHLER 1987; HYNES *et al.* 1986; ROSENBERG *et al.* 1982), and pSymb contains *exo* genes, as well as some metabolic genes (CHARLES and FINAN 1990, 1991).

Early stages of the plant-Rhizobium interaction are characterized by exchange of molecular signals. Flavonoid molecules exuded by the plant roots induce the NodD-dependent expression of *nod* genes (reviewed by LONG 1989a,b; DE BRUIJN and DOWNIE 1991; BREWIN 1991). The bacterial *nod* genes in turn produce a factor to which the plants respond by initiating nodule morphogenesis (LEROUGE *et al.* 1990; SPAINK *et al.* 1991; TRUCHET *et al.* 1991).

nod genes fall into two general categories–the common *nod* genes, such as *nodABC*, and the host specific *nod* genes, such as *nodPQ*, *nodFEG* and *nodH* (reviewed by DÉNARIÉ and ROCHE 1991; LONG 1992). The former are present in all species of Rhizobium, and can substitute for one another across species. The latter do not complement across species even in cases where homologues exist. Mutations in *nodABC* result in a Nod^- phenotype, mutations in *nodH* result in a severe reduction in nodulation ability, and mutations in other host specific *nod* genes result in mild delays of nodulation.

The *nod* genes are required to produce morphogenetic signals sent to the plant roots (FAUCHER *et al.* 1988). *R. meliloti* produces NodRm-1V(S) (formerly called NodRm1), a factor that causes nodulation-re-

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lated reactions on the roots of alfalfa. NodRm-IV(S) is a tetramer of *N*-acetyl glucosamine, with a fatty acyl substitution on one end and a sulfate substitution on the other (LEROUGE *et al.* 1990). A nonsulfated version of the factor is not active on alfalfa roots, but has activity on *Vicia sativa*, a host for *Rhizobium leguminosarum* bv. *viciae* (FAUCHER *et al.* 1989; LEROUGE *et al.* 1990; ROCHE *et al.* 1991). The common *nod* genes are required for the production of any NodRm-IV(S). *nodH* mutants produce only unsulfated factor, and are Nod$^-$ on alfalfa; single *nodQ* mutants produce a mixture of sulfated and unsulfated factor and have a leaky Nod$^-$ phenotype (FAUCHER *et al.* 1988, 1989; LEROUGE *et al.* 1990; ROCHE *et al.* 1991).

We had shown in previous studies that there are two highly conserved copies of *nodPQ* in *R. meliloti*, which would account for the partial phenotypes of single *nodQ* mutants (SCHWEDOCK and LONG 1989), and that *nodP* and *nodQ* together encode ATP sulfurylase (SCHWEDOCK and LONG 1990). This enzyme generates an activated sulfate in the form of adenosine 5′-phosphosulfate (APS) from ATP and SO_4^{2-}. Recent results (our manuscript in preparation) indicate *nodQ* also encodes APS kinase, which synthesizes 3′-phosphoadenosine 5′-phosphosulfate (PAPS) from APS and ATP. NodP and NodQ are homologous with *Escherichia coli cysD*, *cysN* and *cysC* genes that encode the same enzymatic activities in the *E. coli* cysteine biosynthetic pathway (LEYH, TAYLOR and MARKHAM 1988; SATISHCHANDRAN and MARKHAM 1989; SCHWEDOCK and LONG 1990).

The enzymatic studies and the analysis of factor production by mutants are consistent with *nodP* and *nodQ* being involved in the production of NodRm-IV(S). The current model is that NodP and NodQ synthesize PAPS which is then used by NodH for sulfurylation of the Nod-factor precursor (FAUCHER *et al.* 1989; LEROUGE *et al.* 1990; SCHWEDOCK and LONG 1990). In this study we create a double *nodQ* mutant, and show that it has a tight nodulation-defective phenotype (like that of a *nodH* mutant) on alfalfa but it is not a cysteine auxotroph. We show that the second copies of *nodP* and *nodQ* are on pSymb, and present evidence for a third locus in *R. meliloti* that encodes sulfate activating enzymes.

MATERIALS AND METHODS

Strains and media: *E. coli* DH5α (HANAHAN 1985), and HB101 (MANIATIS, FRITSCH and SAMBROOK 1982) have been previously described. SR143 was constructed as follows. pRmJT5 was digested with *Bam*HI and religated in the presence of a spectinomycin resistance (Sp^r) cartridge, such that approximately 15 kb of the original plasmid, from the first *Bam*HI site on the left (*nif* distal, see SWANSON *et al.* 1987; FISHER *et al.* 1987) to the last *Bam*HI site on the right (*nif* proximal), was deleted and replaced with the spectinomycin resistance gene. The Sp^r deletions was homogenotized into Rm1021 to create SR143 (Fig. 1).

FIGURE 1.—Map of region deleted in strain SR143. The dashed line indicates the region of DNA that has been deleted and replaced by a spectinomycin resistance cartridge. *Eco*RI sites are indicated above, and *Bam*HI sites below the line representing pRmJT5. Known genes that are within the deleted region are indicated by arrows above pRmJT5.

Other strains and plasmids used in this study are listed in Table 1 and Table 2, and described in the results. The *R. meliloti* auxotrophs used in Table 2 were kindly provided by JANE GLAZEBROOK and GRAHAM WALKER. pSyma and pSymb are sometimes referred to as pRmeSU47a and pRmeSU47b or pNif and pExo, respectively.

LB (MEADE and SIGNER 1977) and TY (BERINGER 1974), were prepared as previously described. For *R. meliloti*, minimal medium was M9 Sucrose supplemented with biotin (MEADE and SIGNER 1977) and for *E. coli* minimal medium was M9 glucose supplemented with leucine, proline, and thiamine (MANIATIS, FRITSCH and SAMBROOK 1982). Additional supplementations with cysteine and methionine were at 30–40 μg/ml. Antibiotics for *R. meliloti* were used at the following concentrations (μg/ml): tetracycline, 10; neomycin, 50 and 200; gentamycin, 10 and 25; spectinomycin, 50 and 200; streptomycin, 500; and oxytetracycline, 0.5. For *Agrobacterium tumefaciens*: spectinomycin, 100; gentamycin, 25; rifampicin, 50; and nalidixic acid, 50. For *E. coli*: tetracycline, 10; ampicillin, 50; and kanamycin, 25. Where two concentration are indicated, the higher one was used as the selective plates for matings and transductions, and the lower one was used for maintenance of the strains on plates and for growth in liquid media.

Transposable elements are as follows: Tn*5* confers Km^r/Nm^r (BERG and BERG 1987); Tn*5*-233 (DE VOS, WALKER and SIGNER 1986) and Tn*5*-11 (FINAN *et al.* 1986) confer Sp^r/Gm^r; Tn*5*-11 also contains an origin of transfer. Tn*5*-132 confers Ot^r (oxytetracycline resistance) (DE VOS, WALKER and SIGNER 1986).

Plasmid vectors pUC18 (NORRANDER, KEMPE and MESSING 1984), and pUC118 (VIEIRA and MESSING 1987) confer Ap^r; pLAFR1 (FRIEDMAN *et al.* 1982), and pLAFR3 (SWANSON *et al.* 1987) confer Tc^r. Helper plasmid pRK2013 (DITTA *et al.* 1980) confers Km^r. All have been previously described.

Plasmid construction: *Kpn*I-digested pRmS63 was ligated with *Kpn*I-digested pMB50, and the mixture was transformed into DH5a. Tc^r transformants were screened by isolating plasmid DNA via alkaline lysis minipreps (MANIATIS, FRITSCH and SAMBROOK 1982), and checking the DNA with *Kpn*I and *Eco*RI separately. Two isolates, with the 12-kb *Kpn*I fragment in either orientation in pMB50 were saved and named pJSS100 and pJSS101 (see Table 1).

pJSS102-104 (see Table 1) were constructed by ligating the *Kpn*I-*Hin*dIII fragments from pJSS53-55, pUC118 based plasmids, into pMB50. The original fragments cloned into pJSS53-55 were constructed by polymerase chain reaction (SAIKI *et al.* 1985) and sequenced (SANGER, NICKLEN and COULSEN 1977). Clone pJSS104 has two conservative amino acid changes in the predicted NodQ sequence.

Bacterial genetic techniques: Generalized transductions with phage N3 were used throughout to construct strains with insertions of multiple Tn*5* derivatives and to determine genetic linkage (MARTIN and LONG 1984). Distances between markers were estimated using the WU (1966) equa-

TABLE 1

Strains and plasmids

Strain or plasmid	Relevant characteristics or genotype	Source or reference
Rhizobium meliloti[a]		
Rm1021	Str^r derivative of wild type RCR2011	MEADE *et al.* (1982)
A1376	Also known as Rm44d, deletion of pSyma including *nod* and *nif* clusters	M. HONMA
2011-14-6	600-kb pSyma deletion derivative of Rm2011, a different Str^r derivative of RCR2011	M. HYNES
702II	2011-14-6 with $nodQ_2$::Tn*5*#702	This study
JO912	*nodH*::Tn*5*#912	SWANSON *et al.* 1987
JSS12	$nodQ_1$::Tn*5*-233#702	This study
JSS14	$nodQ_2$::Tn*5*#702	This study
JSS16	$nodQ_1$::Tn*5*-233#702 and $nodQ_2$::Tn*5*#702	This study
JSS18	15-kb deletion from SR143 and $nodQ_2$::Tn*5*#702	This study
JSS21	$nodQ_1$::Tn*5*-233#702 and $nodQ_2$::Tn*5*-132#702	This study
JSS27	Deletions from SR143 and RmF117, lacks $nodP_1$, $nodP_2$, $nodQ_1$ and $nodQ_2$	This study
JSS29	Auxotroph, Tn*5* swapped into M3#5 *saa* locus	This study
JSS30	Auxotroph, Tn*5* swapped into DsAux7 *saa* locus	This study
JSS31	*saa*::Tn*5*, $nodQ_1$::Tn*5*-233#702, and $nodQ_2$::Tn*5*-132#702	This study
JSS32	*saa*::Tn*5*-233, *nodH*::Tn*5*#912	This study
JT702	$nodQ_1$::Tn*5*#702	SWANSON *et al.* (1987)
SR143	15-kb deletion in the host specific *nod* gene cluster including $nodQ_1$ and *nodH*	This study
Rm5300	*thi-502*::Tn*5*-11 (on pSymb)	FINAN *et al.* (1986)
Rm5321	Tn*5*-11 (on pSyma)	LONG, MCCUNE and WALKER (1988)
Rm5356	Ω5033::Tn*5*-233	CHARLES and FINAN (1990)
Rm5394	Ω5007::Tn*5*-11	CHARLES and FINAN (1990)
Rm5404	Ω5040::Tn*5*-233	CHARLES and FINAN (1990)
Rm5433	Ω5045::Tn*5*-233	CHARLES and FINAN (1990)
Rm5435	Ω5047::Tn*5*-233	CHARLES and FINAN (1990)
RmF117	ΔΩ5060-5033::Tn*5*-233 (120-kb deletion, including $nodP_2$ and $nodQ_2$)	CHARLES and FINAN (1990)
RmF118	Ω5205::Tn*5*-132	CHARLES and FINAN (1990)
RmF303	Ω5098::Tn*5*-233	CHARLES and FINAN (1990)
RmF351	Ω5111::Tn*5*-233	CHARLES and FINAN (1990)
RmF560	Ω5149::Tn*5*-233	CHARLES and FINAN (1990)
RmF698	Ω5195::Tn*5*-233	CHARLES and FINAN (1990)
M3#5	Cysteine or methionine auxotroph marked with Tn*5*-233 (*saa* locus)	J. GLAZEBROOK and G. C. WALKER
DsAux7	Cysteine or methionine auxotroph marked with Tn*5*-233 (*saa* locus)	J. GLAZEBROOK and G. C. WALKER
Agrobacterium tumefaciens		
A136	Nal^r, Rif^r derivative of NT1, a Ti plasmid cured derivative of C-58	GARFINKLE *et al.* (1981); WATSON *et al.* (1988)
Escherichia coli		
DM62	F$^-$, *cysN96::kan, proC, leu, thi, ara, gal, lac, hsd,str^r*	LEYH, TAYLOR and MARKHAM (1988)
TSL3	*cysD91, strA, recA938::cat*	LEYH, TAYLOR and MARKHAM (1988)
JM81A	F$^-$, *cysC92, tfr8*?	LEYH, TAYLOR and MARKHAM (1988)
HB101		
Plasmids[b]		
pMB50	pLAFR3 with extended polylinker	M. BARNETT (this laboratory)
pRmS63	ColE1 based replicon pUC18 with a 12-kb *Kpn*I fragment containing *nodDABC* through $nodP_1Q_1$	SCHWEDOCK and LONG (1989)
pJSS100	pMB50 with 12-kb fragment of pRmS63 such that the *lac* promoter is near *nodD*	This study
pJSS101	As above, except the *lac* promoter expresses $nodP_1$ and $nodQ_1$	This study
pJSS102	pMB50, expressing $nodP_1$ on a 1.4-kb insert	This study
pJSS103	pMB50, expressing $nodQ_1$ on a 2.1-kb insert	This study
pJSS104	pMB50, expressing $nodP_1$ and $nodQ_1$ on a 3.4-kb insert	This study

(*continued on p. 902*)

TABLE 1

Continued

Strain or plasmid	Relevant characteristics or genotype	Source or reference
pJSS56	ColE1 based replicon pUC118 with a 4.3-kb *Hin*dIII fragment containing $nodP_2$ and $nodQ_2$	This study
pRmJT5	20-kb insert in pLAFR1 containing host range genes	SWANSON *et al.* (1987)
pRmS702	pRmJT5 with Tn*5* insertion #702 in $nodQ_1$	SWANSON *et al.* (1987)

[a] Strains are Rm1021 derivatives unless otherwise indicated.
[b] Plasmids are incP based unless otherwise indicated.

TABLE 2

Complementation of auxotrophs

Strain	Supplement to minimal medium			Plasmid complementation on minimal				
	None	Cys	Met	pJSS100	pJSS101	pJSS102	pJSS103	pJSS104
met83::Tn5	−	−	+	−	−	−	−	−
Rm5003	−	−	+	−	−	−	−	−
Su47met::Tn*5*	−	−	+	−	−	−	−	−
M3#5	−	+	+	−	+	−	−	+
M3#3	−	+/−	+	−	−	−	−	−
DsAux7	−	+	+	−	+	−	−	+
DsAux1	−	+	+	−	−	−	−	−
J5	−	−	+	−	−	−	−	−
GH11	+/−	+	+/−	+/−	+/−	+/−	+/−	+/−
GH6	−	−	+	−	−	−	−	−

− indicates no growth, + indicates robust growth, +/− indicates very slow growth.

tion, $fr = (1 - (x/160))^3$, where fr is frequency of cotransduction, x is distance in kb, and 160 is the estimated size of N3 in kb.

Triparental matings were performed to move pLAFR1 and pLAFR3 based plasmids into *R. meliloti* or endogenous *R. meliloti* megaplasmids into *A. tumefaciens*. These were performed using HB101/pRK2103 as the helper strain, as described by DITTA *et al.* (1980). Homogenotizations of markers on plasmids were performed using plasmid incompatibility techniques as described by RUVKUN and AUSUBEL (1981) with the modifications by JACOBS, EGELHOFF and LONG (1985). Matings to replace one Tn*5* derivative with another by recombination were performed as described by DE VOS, WALKER and SIGNER (1986). Between 1 and 10% of transconjugants from these matings contained the new drug resistance in place of the original drug resistance, indicating replacement.

Southern blot analysis: Agarose Eckhardt gels (ECKHARDT 1978) to separate megaplasmid and chromosome were performed with the modifications of ROSENBERG *et al.* (1982). DNA from both Eckhardt and conventional agarose gels was transferred to Magnagraph nylon membranes (MSI) according to a modification of the technique of REED and MANN (1985), as described by RIGAUD, GRANGE and PICTET (1987). Probes were labeled using the random hexamer priming technique (FEINBERG and VOGELSTEIN 1983). Hybridization conditions were as previously described (SCHWEDOCK and LONG 1989).

Nodulation tests: Alfalfa (*M. sativa*) seeds were surface sterilized and planted on agar slants as described by OGAWA, BRIERLEY and LONG (1991), except that the planting medium was buffered medium Nod3 (EHRHARDT, ATKINSON and LONG 1992). *Melilotus albus* (white sweet clover) seeds were scarified, surface sterilized and planted in vermiculite tubes covered with foil sleeves as described by OGAWA, BRIERLEY and LONG (1991). *V. sativa* subspecies *nigra* (black vetch) seeds were scarified and sterilized using the technique of A. VAN BRUSSEL (personal communication) as follows. The seeds were incubated in concentrated sulfuric acid for 40 min, washed extensively with a large quantity of tap water, soaked in bleach (Clorox) for 10 min, washed six times with sterile water over the course of 6 hr, soaked in sterile water overnight, and then rinsed two more times in fresh sterile water. The seeds were spread on a plate of Nod3 medium plus 1.2% agar, wrapped in foil, and incubated at 4° for at least 7 days, and then planted in vermiculite as above. For both Melilotus and vetch assays (data in Figure 5) only one time point could be taken, as plants must be removed from vemiculite in order to be observed.

Bacterial cultures used for plant inoculations were grown in TY. One milliliter of each culture was washed in 1 ml 10 mM $MgSO_4$ and then diluted into 10 mM $MgSO_4$ to an OD_{600} of 0.01–0.02. One-half milliliter of diluted bacteria was used to inoculate each plant tube.

Vicia nodules were assayed for viable bacteria as follows. Nodules were surface sterilized in 70% ethanol for 5 min,

then in 4% H_2O_2 for 5 min, then washed twice in sterile water for 5 min. The surfaces of the sterilized nodules were rubbed on TY medium to test the thoroughness of sterilization. The nodules were then sliced in half with a sterile razor blade, the inside surfaces were rubbed on TY medium, and the deposited bacteria were streaked out with a toothpick. Colonies formed after 3 days were tested for their drug resistances, ability to grow on various media and morphology.

Size selected library construction: The following method was used to clone $nodP_2Q_2$, which was known to be on a 4.3-kb *Hin*dIII fragment and expected to complement the *E. coli* cysteine auxotroph DM62. *Hin*dIII-digested A1376 DNA was separated by agarose gel electrophoresis, using 0.8% GTG SeaKem agarose (FMC) in 1 × TEA buffer (MANIATIS, FRITSCH and SAMBROOK 1982). Fragments from the 4.2–4.5-kb region of the gel were obtained by the freeze-squeeze technique of TAUTZ and RENZ (1983) and repurified by phenol and chloroform extraction and ethanol precipitation. About 50 ng were ligated with 50 ng *Hin*dIII-digested pUC118 vector in 20 μl, and this mix was transformed into competent DM62 (MANIATIS, FRITSCH and SAMBROOK 1982). Transformation frequency was estimated by plating an aliquot on M9 glucose with ampicillin and cysteine. The remaining cells nine-tenths were plated on M9 glucose with no cysteine, to select for prototrophs. Out of an estimated 1000 colonies, 64 recombinant clones were able to grow in the absence of exogenous cysteine, while none from parallel negative controls showed prototrophy.

RESULTS

Mapping $nodP_2$ and $nodQ_2$ to pSymb: In addition to its chromosome, *R. meliloti* carries two large plasmids, pSyma and pSymb, of approximately 1400 and 1600 kb, respectively (BANFALVI, KONDOROSI and KONDOROSI 1985; BURKARDT and BURKARDT 1984; CHARLES and FINAN 1991). To map the second copies of *nodP* and *nodQ*, we had first to determine their replicon. With an Eckhardt direct lysis gel, the megaplasmids were separated from chromosomal DNA, using strains deleted for the original locus ($nodP_1$ and $nodQ_1$). When filter transfers of this gel were probed with a *nodP* specific probe, we found that the second copy of *nodP* is on one of the Sym plasmids (data not shown).

To determine which megaplasmid carries $nodP_2Q_2$, we separated the two Sym plasmids from one another by conjugation. Rm5321 was used as a donor for pSyma (Tn*5*-11) and Rm5300 as a donor for pSymb (Tn*5*-11) and *A. tumefaciens* strain A136 as the recipient. DNA was isolated from the resulting transconjugants and probed their DNA on a Southern blot. The data in Figure 2 indicate that $nodP_2$ is located on pSymb. In lane 1, DNA from wild type *R. meliloti* shows three bands hybridizing with the *nodP* specific probe. The darkest hybridizing fragment is present in both copies. The fainter upper band is the expected size for the restriction map of the $nodP_1Q_1$ region. The fainter lower band corresponds to the second copy, *i.e.*, the band that remains on Southern blots of DNA from strains deleted for the first copy (SCHWEDOCK and LONG 1989). The upper and middle bands are genetically correlated with pSyma (lane 3), as expected. The middle and lower bands correlate with pSymb (lane 4). Similar results were obtained with a *nodQ* specific probe (data not shown).

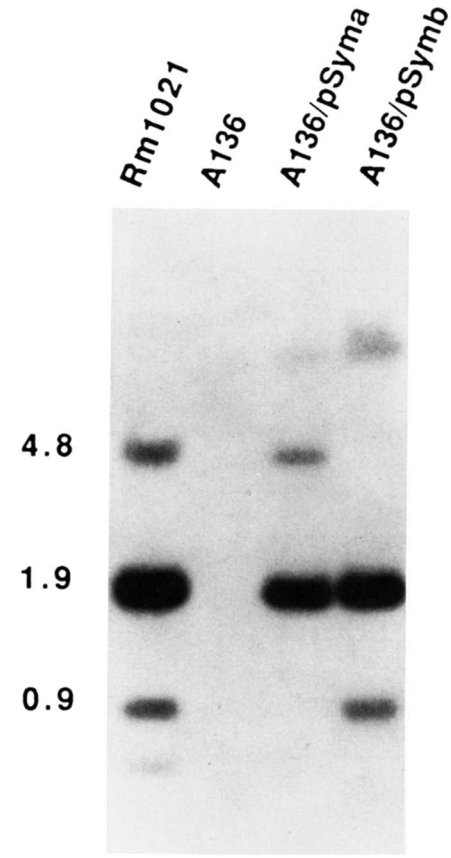

FIGURE 2.—Southern blot hybridization of *R. meliloti* megaplasmids in *A. tumefaciens* with a probe internal to *nodP*. The lanes contain total DNA from *R. meliloti* Rm1021, *A. tumefaciens* A136, A136 with pSyma, and A136 with pSymb, digested with *Xho*I. The numbers to the left indicate the estimated sizes of the hybridizing fragments in kb.

To map the locus more precisely on pSym-b, a strain was constructed that had a marker at the second copy of *nodQ*. The copies of *nodP* and *nodQ* on the two Sym plasmids are highly homologous, as indicated by the intensity of cross hybridization on Southern blots and their apparently conserved restriction enzyme map (SCHWEDOCK and LONG 1989) (data shown below); we took advantage of this sequence similarity to mark $nodQ_2$. Plasmid pRmS702, containing Tn*5* insertion #702 in $nodQ_1$, was introduced into 2011-14-6, a strain deleted for $nodQ_1$ and a large portion of pSyma. The Tn*5* insertion from pRmS702 was then marker exchanged into the $nodQ_2$ locus on pSymb, producing strain 702II. The $nodQ_2$ Tn*5* insertion was then transduced into Rm1021 to create JSS14, a strain with a normal $nodQ_1$ and a mutation in $nodQ_2$. The large size (160 kb) of the segment transferred by the transducing phage ensured that recombination occurred at pSymb.

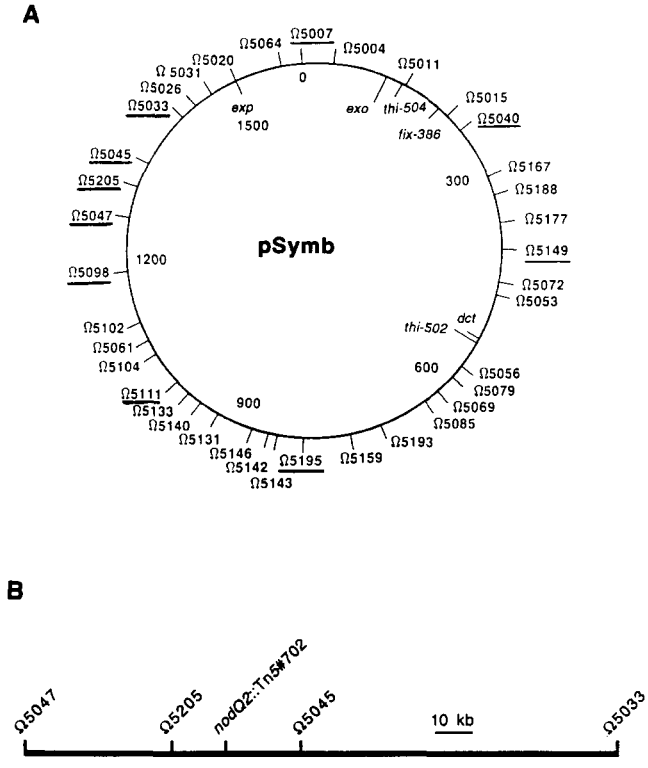

FIGURE 3.—Map of markers on pSymb. (**A**) Map of the entire megaplasmid, reprinted from CHARLES and FINAN (1990). Positions that were used to map *nodQ*$_2$::Tn*5*-233#702 are underlined. (**B**) Position of *nodQ*$_2$::Tn*5*-233#702 with respect to nearby markers on an expanded map.

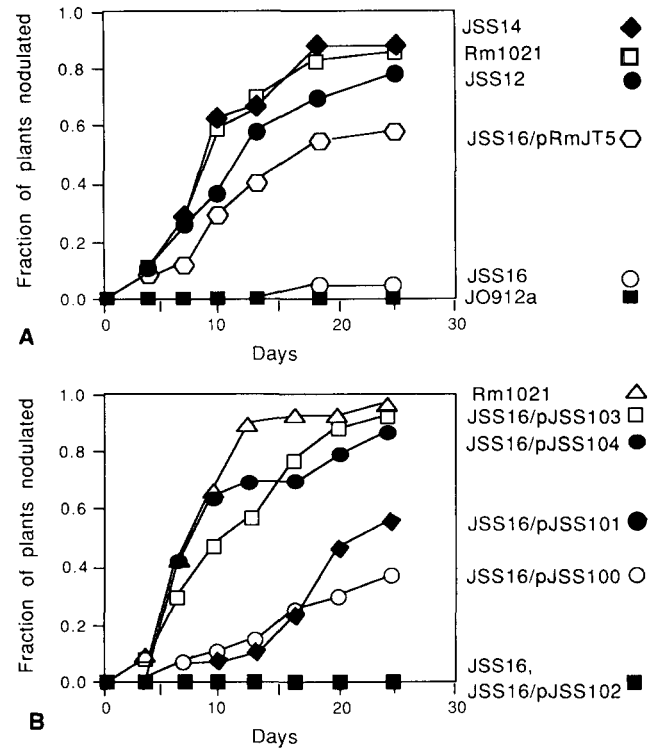

FIGURE 4.—Nodulation time course for various *R. meliloti* mutants on alfalfa. (**A**) Phenotypes of single and double *nodQ*, and *nodH* mutants. (**B**) The double *nodQ* mutant complemented with various subclones of *nodP*$_1$ and *nodQ*$_1$. The plants were inoculated on day zero. Eighteen to 22 plants were used for each trial. The number of plants with at least one nodule divided by the number of plants in the trial equals the fraction of plants nodulated. The strains are indicated at the right of their plots.

The Tn*5* marker was used to test *nodQ*$_2$ for linkage to a set of widely spaced markers. First, the Nm^r from JSS14 was transduced into six strains, each carrying a mapped Tn*5*-233 insertion in pSymb, at Ω5033, 5007, 5040, 5111, 5149 or 5195 (underlined, Figure 3A). We screened the transductants for the loss of Sp^r, which would indicate linkage to the Tn*5*-233 position. The Tn*5*-233 at Ω5033 (strain Rm5356) is linked by 4.4% to the Tn*5* of JSS14 in *nodQ*$_2$, indicating that these markers are about 103 kb apart as calculated by the Wu equation (MARTIN and LONG 1984; CHARLES and FINAN 1990). We repeated the procedure with strains carrying near inserts Ω5033, namely Ω5045, 5047, 5205 and 5098 (Figure 3) and found that *nodQ*$_2$::Tn*5*#702 is 14 kb from Ω5205 and 21 kb from Ω5045 (Figure 3B).

Growth characteristics of mutants in *nodQ*$_1$ and *nodQ*$_2$: Because *nodP* and *nodQ* are homologous to *E. coli cysD* and *cysN* (SCHWEDOCK and LONG 1990), we expected that the double *nodQ*::Tn*5* derivative mutant would be a cysteine auxotroph. This was not the case, however. The double mutant, JSS16, *nodQ*$_1$::Tn*5*-233, *nodQ*$_2$::Tn*5*-132 was able to grow on minimal medium, though less well than wild type. The addition of cysteine decreased colony size further for the *nodQ*$_1$*nodQ*$_2$ double mutant, whereas the addition of methionine resulted in a colony size near that of wild type. The basis for this is not yet known. To rule out the possibility that the prototrophy of JSS16 resulted from residual activity of truncated *nodQ* products, we tested JSS27, a strain physically deleted for both *nodP*$_1$*Q*$_2$ and *nodP*$_2$*Q*$_2$, and found it was also prototrophic. Assuming sulfur activation steps in cysteine biosynthetic pathways to be conserved, these results strongly imply the existence of other structural genes for the enzymes that generate PAPS.

Nodulation phenotypes of mutants in *nodQ*$_1$ and *nodQ*$_2$: The original mutants with Tn*5* insertions in *nodP*$_1$ or *nodQ*$_1$ were delayed in nodulation on alfalfa (*M. sativa*) (DEBELLÉ *et al.* 1986; SWANSON *et al.* 1987). Their phenotype was often difficult to distinguish from that of wild-type Rm1021 (Figure 4A). The double *nodQ*$_1$*nodQ*$_2$ mutant, JSS16, was severely impaired in its ability to nodulate alfalfa, in contrast to either single mutant, though it had a slightly better ability to nodulate alfalfa than JO912a, the *nodH* mutant (Figure 4A). *nodH* and double *nodQ* mutants are reported to make only unsulfated factor (FAUCHER *et al.* 1988, 1989; LEROUGE *et al.* 1990; ROCHE *et al.* 1991). We found that the nodulation ability of the *nodQ*$_1$*nodQ*$_2$ mutant, about 5% of plants overall, was

always equal to or greater than that of a *nodH* mutant, about 1%.

We observed that low copy number recombinant plasmid pRmJT5 can complement the nodulation phenotype of JSS16 (*nodQ*$_1$::Tn*5*-233, *nodQ*$_2$::Tn*5*-132) on alfalfa, although not quite to wild-type levels (Figure 4A). We constructed appropriate subclones and found that plasmids containing *nodQ*$_1$ only (pJSS103), or *nodP*$_1$ and *nodQ*$_1$ only (pJSS104), fully complement the nodulation defect of JSS16 (Figure 4B). The plasmid with only *nodP*$_1$ (pJSS102) did not complement the *nodQ*$_1$*nodQ*$_2$ mutant, and plasmids containing other *nod* genes in addition to *nodP*$_1$ and *nodQ*$_1$ (pJSS100 and 101) only partially complemented the nodulation phenotype, and caused poor growth and a mucoid phenotype. Thus, *nodQ*$_1$ alone was sufficient to complement the nodulation phenotype of JSS16, and that the overexpression of other *nod* genes decreased the effectiveness of this complementation.

On a different homologous plant host, *M. albus*, the double *nodQ*$_1$*Q*$_2$ mutant was only slightly impaired in nodulation, and was similar to JSS12, the *nodQ*$_1$ single mutant (Figure 5A). Its phenotype was also similar to that for a *nodH* mutant (OGAWA, BRIERLEY and LONG 1991).

Because the unsulfated Nod factor is active on the roots of *V. sativa* (vetch) (FAUCHER *et al.* 1989; LEROUGE *et al.* 1990), we tested the ability of *nodQ* mutants to nodulate this heterologous host plant. The double *nodQ* mutant, JSS16, nodulated vetch well, as did JSS12 (*nodQ*$_1$) and JO912a (*nodH*) (Figure 5B). Wild-type Rm1021, and the single *nodQ*$_2$ mutant, JSS14, only nodulated between 10 and 20% of the vetch plants (Figure 5B). Thus in our hands the host range system is less stringent than in the observations of FAUCHER *et al.* (1988) who reported that wild-type *R. meliloti* did not nodulate vetch at all. The nodules formed by R8401/pRL1 (Figure 5B), a *R. leguminosarum* bv. *viciae* strain, were pink and yielded numerous colonies from the nodule interior, while the nodules from the various *R. meliloti* strains were small and white indicating they were not able to fix nitrogen. We found no viable bacteria inside these nodules.

Complementing *R. meliloti* auxotrophs with *nodP*$_1$ and *nodQ*$_1$: Because the *nodQ*$_1$*Q*$_2$ double-null mutants (Tn*5* insertions or deletions) were not auxotrophic, we inferred the existence of a third locus, encoding enzymes that form PAPS for cysteine biosynthesis, and searched for it using ten *Cys* or *Met* auxotrophs. Plasmids containing *nodP*$_1$ and *nodQ*$_1$ driven by the *lac* promoter were introduced and tested for complementation, in order to define auxotrophic mutants defective in PAPS formation. Two mutants were complemented by *nodP*$_1$ and *nodQ*$_1$ together on a plasmid ((DsAux7 and M3#5 with pJSS101 and 104; see Table 2). The mutants were not complemented by a plasmid containing either gene alone (pJSS102 and 103), or containing *nodP*$_1$ and *nodQ*$_1$ in the wrong orientation with respect to the *lac* promoter (pJSS100). We replaced Tn*5* in DsAux7 and in M3#5 with Tn*5*-233 to obtain a different resistance marker, and by reciprocal transductions showed that the two are closely linked (10/108 separated, indicating linkage within 0.5 kb). We used JSS30, one of the Cys$^-$ auxotrophs in which Tn*5*-233 was replaced with Tn*5*, to establish cotransduction of the insertions with the auxotrophic phenotype. We have named the locus *saa* for *s*ulfur *a*mino *a*cid, as we found that either cysteine or methionine rescues the mutant.

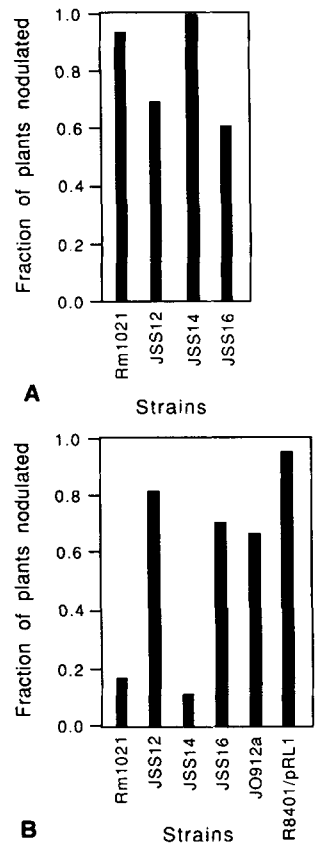

FIGURE 5.—Nodulation of *M. albus* (**A**) and vetch (**B**) by single and double *nodQ* mutants. The plants were grown in vermiculite and dug up 21 days after inoculation. Twelve to 16 plants were used for each *M. albus* trial and 16–20 plants were used for each vetch trial. The number of plants with at least one nodule divided by the number of plants in the trial equals the fraction of plants nodulated.

We constructed a triple mutant, *saa*, *nodQ*$_1$, *nodQ*$_2$, to show that *saa* is a distinct locus, and to test whether the strain was viable. The *saa*::Tn*5* mutation of JSS30 was transduced into a *nodQ*$_1$::Tn*5*-132, *nodQ*^{2}::Tn*5*-233 strain, creating JSS31. This triple mutant is viable on LB though it grows more slowly than JSS21 or JSS30. It was, as expected, an auxotroph requiring either methionine or cysteine.

Since *nodPQ* appeared to be functionally redundant

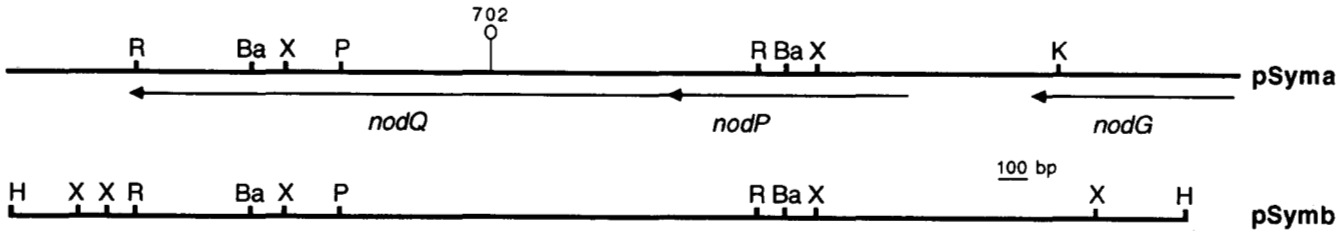

FIGURE 6.—Comparison of the restriction maps of the two *nodPQ* loci. The upper map is of the $nodP_1Q_1$ region on pSyma. The positions of $nodP_1$, $nodQ_1$ and *nodG* are indicated by the arrows. The position of Tn*5* insertion #702 is indicated by (O). The lower map is the region cloned from pSymb that corresponds to $nodP_2Q_2$. Restriction enzyme sites are as follows: H, *Hin*dIII; X, *Xho*I; R, *Eco*RI; Ba, *Bam*HI; P, *Pst*I; K, *Kpn*I.

with *saa*, we wondered why *nodPQ* function did not mask the phenotype of the *saa*::Tn*5* mutation. Possible explanations could be either that *nodPQ* is not expressed sufficiently to generate sufficient enzyme; that the *nodPQ* encoded enzymes are much less active than those encoded by *saa*; or that the PAPS generated by gp (*nodPQ*) activity is not generally available to enzymes of amino acid biosynthesis.

We tested the first possibility by activating *nod* gene expression with the plant inducer luteolin. Expression was induced with 3 μM luteolin, a 10-fold excess from the level known to cause maximal *nod* gene induction. Even at this level, *nodPQ* expression does not rescue prototrophic growth of an *saa* mutant strain.

Cloning of $nodP_2$ and $nodQ_2$: To clone the second copy of *nodP* and *nodQ* we took advantage of the fact that the original *nodP* and *nodQ* genes complement the *cysD* mutation in DM62 (SCHWEDOCK and LONG 1990). Southern blot analysis showed that $nodP_2$ and $nodQ_2$ are on a 4.3 kb *Hin*dIII fragment (data not shown). We made a size selected *Hin*dIII library in pUC118 using DNA from a $nodP_1Q_1$ deletion derivative of Rm1021. The library was transformed into DM62. Sixty-four Cys^+ colonies grew up (out of an estimated 1000 recombinant-containing transformants), and half were analyzed for plasmid DNA content. Thirty of these had two identical *Hin*dIII fragments of identical size, representing the vector and the insert. The remaining two clones had the same fragments as the others plus an additional one. Two of the 30 apparently identical clones were chosen for further study. Both of these complemented all three different *E. coli* Cys^- mutants, as had been true of $nodP_1nodQ_1$ clones (SCHWEDOCK and LONG 1990).

By restriction enzyme analysis (Figure 6), we determined that the map of one clone, designated pJSS56, was consistent with our Southern blot analysis of the second copy (SCHWEDOCK and LONG 1989) (data not shown). The first restriction site divergence between $nodP_1Q_1$ and $nodP_2Q_2$ was found to occur within 500 bp of the start of *nodP* and within 100 bp of the end of *nodQ*.

DISCUSSION

It is not unusual for *R. meliloti* to have more than one copy of a gene (reviewed by LONG 1989b, 1992). For example, there are three loosely linked copies of the regulatory gene *nodD*, each of which alone is sufficient for some nodulation. *R. meliloti* also has at least two copies of *ftsZ* (MARGOLIN, CORBO and LONG 1991) and at least two copies of *GroESL* (J. OGAWA and S. LONG, unpublished). The $nodP_1Q_2$ and $nodP_2Q_2$ genes appear to be highly homologous, since for all the restriction enzymes tested, sites falling within the open reading frames are conserved. This high homology breaks down in the flanking DNA (this paper and M. WILLITS and S. R. LONG, unpublished data). The functional redundancy of the *nodPQ* loci accounts for the weak phenotypes of single mutants. In fact, many of the strains with Tn*5* insertions in $nodP_1$ or $nodQ_1$ alone had no significant difference from wild type in their ability to nodulate alfalfa (SWANSON *et al.* 1987). However, the double *nodQ* mutant, while not completely Nod^-, showed reduced and delayed nodulation on alfalfa.

The nodulation phenotype of the double $nodQ_1Q_2$ mutant is similar to that of *nodH* mutants. NodP and NodQ are proposed to be enzymes involved in the pathway of NodRm-IV(S) biosynthesis, as is NodH (ROCHE *et al.* 1991). NodP and NodQ make an activated sulfate group (PAPS) (SCHWEDOCK and LONG 1990; and our manuscript in preparation), which is presumably used to sulfurylate the NodRm-IV(S) precursor. Without these enzymes, *R. meliloti* makes an unsulfated version of the factor, which is no longer active on alfalfa (FAUCHER *et al.* 1988, 1989; LEROUGE *et al.* 1990; ROCHE *et al.* 1991). Under the conditions used in this study, *nodH* mutants occasionally induce the formation of a nodule (on about 1% of alfalfa plants) (OGAWA, BRIERLEY and LONG 1991; SWANSON *et al.* 1987). JSS16, a double *nodQ* mutant, is also very defective for symbiosis, showing nodulation of about 5% of the plants.

We propose that there is yet a third locus that activates sulfate. The evidence for this is as follows. First, strains in which all *nodP* and *nodQ* genes are deleted or otherwise mutated are still prototrophic for cysteine and methionine. These mutants must have structural genes elsewhere encoding sulfate activation, the required initial step for cysteine and

methionine biosynthesis. We have confirmed that the double mutants still have ATP sulfurylase activity (our manuscript in preparation). Second, we found a methionine or cysteine requiring auxotrophic mutation that can be complemented by the overexpression of $nodP_1$ and $nodQ_1$. This mutation is apparently not linked to the *nodPQ* loci. We infer that this locus, *saa*, either encodes the enzymatic activity for PAPS synthesis, or a regulatory gene.

The heterologous host plant, vetch, responds to nonsulfated Nod factor (ROCHE *et al.* 1991). We found that a defect in $nodQ_1$ (for example, JSS16 and JSS12) caused strains to have higher nodulation of vetch, as, conversely, a $nodQ_1$ mutation caused a decrease in alfalfa nodulation, for which sulfated factor is required. The $nodQ_2$ locus appears less important for either phenotype. From this we infer that the $nodQ_1$ locus is responsible for the production of the majority of the sulfated Nod factor. We noted that even while $nodQ_1$ mutants nodulate vetch fairly well, they are not able to invade the nodules efficiently. Possibly, the unsulfated version of an *R. meliloti* factor may be similar enough to a *R. leguminosarum* bv. *viciae* factor to allow initial organogenesis to occur on vetch, but differences in the fatty acyl group (an 18:4 chain in *R. leguminosarum* bv. *viciae* (SPAINK *et al.* 1991) as opposed to the 16:2 chain of *R. meliloti*) may not allow invasion of bacteria into the plant. This may be testable by changing the *nodFE* genes of *R. meliloti*. Alternatively, if surface markers or exopolysaccharides (EPS) determine invasion, then while the Nod factor of the mutant *R. meliloti* causes organogenesis on vetch, incompatibilities on cell surfaces may block further steps.

In *E. coli*, PAPS is used in the cysteine biosynthetic pathway, and then the sulfur from cysteine is used in methionine biosynthesis (COHEN and SAINT-GIRONS 1987; KREDICH 1987). In yeast PAPS is used directly in methionine as well as in cysteine biosynthesis (ROSE and HARRISON 1971). Yeast can also make methionine from cysteine and vice versa via cystathionine (ROSE and HARRISON 1971). *R. meliloti saa* mutants grow on either cysteine or methionine, unlike *E. coli cysDNC* mutants (KREDICH 1987; LEYH, TAYLOR and MARKHAM 1988). The *saa* mutants, which are specifically defective in PAPS synthesis, are unlike the Cym^- mutants reviewed by KREDICH (1987), which appear to be regulatory or pleiotropic. The pathways for *R. meliloti* amino acid synthesis need to be worked out in order to understand how cysteine and methionine relate to the initial steps of sulfate activation.

What are the total requirements of *R. meliloti* for PAPS? Around this central question cluster a number of related ones. The $nodP_1Q_1$ locus is linked to *nod* genes involved in production of a sulfated factor; expression and complementation data suggest the PAPS requirement for Nod factor synthesis is less than the PAPS requirement for cysteine and methionine synthesis. For example, a $nodP_1Q_1$ clone under its own promoter suffices to complement a $nodP_1Q_1$-$nodP_2Q_2$-mutant for Nod factor production, but a higher level exogenous plasmid promoter is required for *nodPQ* to restore growth to an *saa* mutant. The logical corollary of this is that the *saa* PAPS synthesis pathway required for amino acid production provides a higher level of PAPS; thus one open question is why this PAPS does not suffice for Nod factor sulfation. Regulation, enzyme activity, and/or metabolite channeling are possibilities that should be considered. Further study will be possible with cloning of the *saa* locus and analysis of its expression and its protein products.

The $nodP_2Q_2$ locus, which we mapped to pSymb, provides another question. As functionally related genes are often clustered, we note that many genes for EPS and some for lipopolysaccharide (LPS) synthesis are located on plasmids (reviewed by LONG 1989b) and that *R. meliloti* LPS is sulfurylated (R. HOLLINGSWORTH, personal communication). It is possible the $nodP_2Q_2$ encodes PAPS synthetic functions for use in sulfurylating LPS or other extracellular products. An initial possibility to test is whether the $nodP_2Q_2$ genes are co-regulated with genes for EPS and/or LPS synthesis. Finally, with respect to *saa* and *nodPQ* function, an interesting question will be how the protein products themselves are regulated. We have shown (our manuscript in preparation) that the NodP-NodQ complex requires GTP for activity. It will be interesting to test the other PAPS synthesis enzymes for GTP dependence, and to ask what coordinating or regulatory role may be played by GTP in *R. meliloti* sulfate metabolism.

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